Epidemiol. Infect. (2015), **143**, 2373–2380. © Cambridge University Press 2014
doi:10.1017/S0950268814003410

Genotypes and antibiotic resistance of bovine *Campylobacter* and their contribution to human campylobacteriosis

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Received 14 August 2014; Final revision 13 November 2014; Accepted 19 November 2014; first published online 16 December 2014

SUMMARY

Campylobacter jejuni and *Campylobacter coli* are the most important bacterial causes of human gastroenteritis. Chicken has been recognized as a major source for human infection, whereas cattle might also contribute to a lesser extent. However, there is a paucity of information available regarding *Campylobacter* in Swiss cattle and their role for human campylobacteriosis. To gain more information on genotypes and antibiotic resistance of bovine *C. jejuni* and *C. coli* and on their contribution to human disease, 97 cattle isolates were analysed. Multilocus sequence typing (MLST) and *flaB* typing were applied and the *gyrA* and 23S rRNA genes were screened for point mutations responsible for quinolone and macrolide resistance, respectively. A total of 37 sequence types (STs) and 44 *flaB* types were identified, including two sequence types and five *flaB* types not previously described. Most common sequence types were ST21 (21%), ST61 (12%) and ST48 (11%). Only one isolate was macrolide resistant while 31% ($n = 30$) were quinolone resistant. Source attribution indicated chicken as the main source of human infection with cattle being second. In conclusion, cattle should not be underestimated as a potential source of human campylobacteriosis.

Key words: Antibiotic resistance, *Campylobacter coli*, *Campylobacter jejuni*, *flaB*, gastroenteritis, genoptyping, MLST, source attribution, zoonosis.

INTRODUCTION

Campylobacteriosis is the most commonly reported zoonosis in Switzerland and the European Union. In 2012, 214268 confirmed cases of campylobacteriosis in humans were reported in the European Union, but estimates are as high as 9 million cases [1, 2]. *Campylobacter* infections usually cause enteric symptoms, including diarrhoea (frequently with blood), abdominal pain, fever, headache and nausea (sometimes with vomiting). In most cases the disease is self-limiting with symptoms only lasting 3–6 days and then stopping even without treatment. However, in some cases severe complications such as Guillain–Barré syndrome or reactive arthritis can develop [3, 4]. About 90% of human *Campylobacter* infections are due to *Campylobacter jejuni*, with *C. coli* being responsible for most of the remaining cases [5].

C. jejuni and *C. coli* are commensals in the gastrointestinal tract of many food-production animals, but they may also be found in pets and the environment [6–8]. Infection can therefore occur by consumption of undercooked chicken meat, unpasteurized milk, by contaminated environmental sources and contact with pets and farm animals [2]. Affected adult animals are usually not sick but they shed the bacteria in their faeces, thus, playing a central role as a reservoir [4]. According to a study in the UK, 21% of cattle shed

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Campylobacter in faeces with 97·7% and 2·3% thereof being *C. jejuni* and *C. coli*, respectively [9]. In Switzerland in 2012 only 12·8% of cattle at slaughter were found *Campylobacter* positive of which 79·2% were *C. jejuni* and 20·8% were *C. coli* [10].

With regard to meat from chicken and cattle or milk, the prevalence of *Campylobacter* in beef is generally much lower (3·2%) compared to broilers (49·9%) and even lower in raw milk (1·6%), e.g. as shown for Ireland [11]. By contrast, for Northern Italy the prevalence of *Campylobacter* in raw milk was estimated at 12% [12].

Source attribution studies have indicated chickens as the most important source of human campylobacteriosis [13–19]. This is also illustrated by the effect the Belgian dioxin crisis had in 1999. It clearly showed the relationship between chicken consumption and campylobacteriosis in humans with a decline of 40% of campylobacteriosis cases when chicken was taken off the market [20]. Moreover, cattle may be a significant reservoir for human cases [21, 22].

Standardized and highly reproducible multilocus sequence typing (MLST) schemes have been established for *C. jejuni and C. coli* [23, 24]. MLST ensures a uniform nomenclature with defined sequence types (STs) and clonal complexes (CCs) that allows for population studies. Korczak *et al.* [25] optimized, simplified and unified MLST by multiplexing and using a minimal set of primers for amplification and sequencing. By adding *flaB* typing, a further distinction of identical STs could be achieved. Finally, genetic determination of antibiotic resistance against macrolides and quinolones was included in the optimized typing scheme. These resistances can be assessed by detecting point mutations in the *gyrA* (C257T) gene or in the 23S rRNA gene (A2074G or A2075G), which are responsible for quinolone and macrolide resistance, respectively [26].

Up to now, no comprehensive studies regarding genotypes and antibiotic resistance of *C. jejuni* and *C. coli* have been conducted in Swiss cattle. Therefore, MLST, *flaB* typing and sequence-based determination of macrolide and quinolone resistance were used to characterize *C. jejuni* and *C. coli* in Swiss cattle and to determine their possible role as a reservoir of human infection.

MATERIAL AND METHODS

Strains and DNA preparation

A total of 97 *C. jejuni* and *C. coli* isolates were investigated. They included 78 isolates from healthy cows collected at slaughterhouses between 2008 and 2012 for resistance monitoring by the Federal Food Safety and Veterinary Office (FSVO), and 19 strains received from diagnostic submissions of diarrhoeic cattle suspected of salmonellosis at the Institute of Veterinary Bacteriology, Bern between January 2013 and March 2014. The isolates were stored at −80 °C until cultivation on tryptone soya agar plates with sheep blood (TSA; Becton Dickinson AG, Switzerland) for 48–72 h at 42 °C under microaerophilic conditions.

DNA template preparation was achieved using a simple lysis method. A few colonies were picked from each plate and added to 500 μl lysis buffer (0·1 m Tris–HCl, pH 8·5, 0·05% Tween-20, 240 μg/ml proteinase K), then incubated for 1 h at 60 °C followed by 15 min at 95 °C. Lysates were directly used or stored at −20 °C.

Genotyping

MLST, *flaB* typing as well as determination of macrolide and quinolone resistance based on partial sequences of 23S rRNA and *gyrA* genes, respectively, was performed according to Korczak *et al.* [25]. Sequences were edited and analysed using the SmartGene® Campylobacter MLST platform (SmartGene, Switzerland) including a direct link to the PubMLST database (www.pubmlst.org) to automatically determined the allele number, ST and CC. The *flaB* sequences were directly queried on PubMLST to determine allele numbers. The 23S rRNA gene fragments were screened for the A2074G and A2075G point mutations and the *gyrA* gene fragments were checked for the C257T mutation.

Statistical analysis

Proportions and 95% confidence intervals (CIs) were calculated with the exact binomial model in NCSS9 software (NCSS, USA). Simpson's Index (also known as the discriminatory index) was calculated according to Hunter & Gaston [27]. Possible associations between genotypes and quinolone resistance were examined with Pearson's χ^2 test to check the null hypothesis that genotypes and quinolone resistance are independent. The significance level was set at $P \leqslant 0{\cdot}05$. The same approach was used to examine the association between *Campylobacter* sp. and resistance.

Population analyses and source attribution

To assess the similarity of Swiss *Campylobacter* populations the proportional similarity index (PSI) was

Table 1. *Distribution of clonal complexes (CC), sequence types (ST),* flaB *types and antibiotic resistance in* C. jejuni *and* C. coli *isolates from cattle*

Species	CC	ST	*flaB*	No.of isolates	No. of resistant isolates (macrolide/ quinolone)
C. jejuni	21	19	36	4	0/1
		21	78	1	0/1
		21	103	3	0/2
		21	198	9	0/6
		21	245	1	0/0
		21	371	2	0/2
		21	414	2	0/0
		21	1631	1	0/0
		21	1641	1	0/0
		50	36	1	0/0
		262	137	1	0/0
		262	1642	1	0/0
		262	1643	1	0/0
	22	22	442	1	0/1
	42	42	177	3	0/0
		42	440	1	0/0
	45	45	463	1	0/0
		334	177	1	0/0
		137	441	1	0/0
	48	48	103	10	0/2
		48	1644	1	0/1
		844	36	1	0/0
	52	52	57	1	0/1
	61	61	42	1	0/0
		61	1179	10	0/0
		61	339	1	0/0
		2341	1179	1	0/0
	206	122	47	1	0/0
		572	260	1	0/0
		572	96	1	0/1
	257	257	16	1	0/0
		257	301	2	0/0
	403	1775	51	1	0/0
	n.d.	586	1640	1	0/0
		586	402	1	0/1
		6264	34	1	0/1
		7135	371	1	0/0
C. coli	828	827	236	3	0/1
		854	915	2	0/1
		854	13	1	0/0
		854	17	1	0/1
		854	319	1	0/0
		854	528	2	0/0
		1096	13	1	1/1
		1413	125	1	0/1
		2709	667	1	0/0
		3023	500	1	0/1
		3336	13	1	0/0
		4946	660	1	0/0
		7134	500	1	0/0
	n.d.	1049	310	1	0/0
		1680	633	1	0/0
		3345	500	1	0/1
		4936	721	1	0/1
		4948	500	1	0/1
		4953	500	1	0/0
		4962	13	1	0/1
Total				97	1/30

n.d., Indicates STs for which no CC is defined.

calculated as described previously [28]. Genotypes of cattle *C. jejuni* and *C. coli* isolates based on MLST and *flaB* were compared with 383 human *C. jejuni* and *C. coli* isolates from cases without a record of foreign travel collected in 2009 [5], 197 chicken *C. jejuni* and *C. coli* isolates collected in 2009 [29], 134 dog *C. jejuni* isolates collected between 2003 and 2012 [30] and 256 pig *C. coli* isolates collected in 2009 [31]. In addition, the genetic distances between the Swiss *Campylobacter* populations from different sources were estimated by calculating fixation indices (F_{st}) using the concatenated sequences of the seven MLST loci or the *flaB* sequences, employing Arlequin software [32]. To assign human isolates to their most probable source based on either the MLST alleles or the *flaB* sequence STRUCTURE software (http://pritchardlab.stanford.edu/structure.html) was used as described previously except that the MIGRPRIOR parameter was set to zero to provide a better separation between the source clusters [19, 33].

RESULTS

Genotyping

Complete MLST and *flaB* sequence data was obtained from the 97 investigated isolates, comprising 75% *C. jejuni* ($n = 73$, 95% CI 66–84) and 25% *C. coli* ($n = 24$, 95% CI 17–35). A total of 37 different STs were identified in the samples, two of which were new (Table 1). These were submitted to the PubMLST database for number assignment. One of the STs was a previously unreported combination of alleles in *C. jejuni* (ST7135) and the other, a new allele sequence for *glmM* (allele 703)

Table 2. *Proportional similarity index (PSI) of* C. jejuni *and* C. coli *isolates from different sources*

	MLST		*flaB*	
	Cattle	Human	Cattle	Human
C. jejuni				
Cattle	1		1	
Human	0·54 (0·46–0·6)	1	0·53 (0·43–0·63)	1
Chicken	0·44 (0·34–0·54)	0·58 (0·50–0·65)	0·42 (0·31–0·52)	0·66 (0·59–0·72)
Dog	0·38 (0·30–0·46)	0·42 (0·35–0·49)	0·41 (0·32–0·50)	0·52 (0·44–0·61)
C. coli				
Cattle	1		1	
Human	0·22 (0·06–0·38)	1	0·22 (0·06–0·38)	1
Chicken	0·36 (0·20–0·51)	0·41 (0·26–0·56)	0·43 (0·28–0·59)	0·47 (0·31–0·63)
Pigs	0·36 (0·23–0·48)	0·10 (0·03–0·17)	0·47 (0·32–0·62)	0·06 (0·00–0·12)

Values within parentheses are 95% confidence intervals.
1 = maximal similarity; 0 = maximal difference.

resulting in the new ST7134 in *C. coli*. The most common STs were ST21 (21%, $n = 20$), ST61 (12%, $n = 12$), ST48 (11%, $n = 11$) and ST854 (7%, $n = 7$). Twenty-six of the 37 STs were represented by single isolates. The STs were distributed over 11 CCs. The most common CCs were CC21 (29%, $n = 28$), CC828 (18%, $n = 17$), CC61 (13%, $n = 13$) and CC48 (12%, $n = 12$). The group termed 'Not defined' contained ten STs (11%) not associated with any CC.

The analysis of *flaB* sequences showed 44 different types, five of which had not been described previously (types 1640–1644). The most common *flaB* types were 103 (13%, $n = 13$), 1179 (11%, $n = 11$), 198 (9%, $n = 9$) and 36 (6%, $n = 6$). All *flaB* type 198 belonged to CC21 and all *flaB* type 1179 belonged to CC61, whereas for *flaB* type 103, three isolates belonged to CC21 and ten isolates to CC48.

Simpson's Index was 0·92 for MLST, 0·95 for *flaB* typing and 0·97 for the combination of both methods.

Antibiotic resistance

The majority (69%, $n = 67$) of isolates were sensitive to quinolones and 31% ($n = 30$) of isolates were resistant based on the corresponding mutation in the *gyrA* gene. At the species level 42% ($n = 10/24$) of *C. coli* and 27% ($n = 20/73$) of *C. jejuni* were resistant to quinolones. There was only a single *C. coli* isolate showing resistance towards macrolides based on mutation A2075G. This isolate was also resistant towards quinolones. The percentage of quinolone-resistant strains within the most common CCs was 43% ($n = 12/28$) in CC21, 35% ($n = 6/17$) in CC828, 25% ($n = 3/12$) in CC48 and no resistant strains ($n = 0/13$) were observed in CC61. No association was found between *Campylobacter* sp. and quinolone resistance ($P = 0·19$). CC61 was significantly more often sensitive towards quinolones compared to the general resistance distribution ($P = 0·02$), whereas the other CCs did not differ significantly from the overall quinolone resistance distribution.

Population analyses and source attribution

PSI

The PSIs were calculated for populations based on MLST and *flaB* typing. Values were calculated separately for *C. jejuni* and *C. coli*. As shown in Table 2, *C. jejuni* cattle isolates showed the highest overlap with human isolates followed by chicken and dog isolates independent of the typing scheme. For *C. coli* the overlap between cattle and pigs was highest with the *flaB* genotyping method whereas with MLST the overlap between cattle and pigs was as high as between cattle and chicken. In any case human isolates showed highest overlap with chicken independent of genotyping scheme and *Campylobacter* sp. (Table 2).

F_{st} *analysis*

When using MLST sequences, the genetic distance based on fixation indices (F_{st}) between all *Campylobacter* host groups differed significantly from zero (Table 3). Based on MLST data cattle isolates were closest to chicken *C. jejuni* and porcine *C. coli* isolates. Using the *flaB* typing method cattle isolates were most similar to canine *C. jejuni* isolates

Table 3. *Fixation indices (F_{st}) for* C. jejuni *and* C. coli *isolates from different sources*

	F_{st} MLST				F_{st} *flaB*			
	Cattle	Dog	Human	Chicken	Cattle	Dog	Human	Chicken
C. jejuni								
Cattle	0				0			
Dog	0·07 (0·06–0·08)	0			0·02 (0·01–0·02)	0		
Human	0·06 (0·05–0·07)	0·10 (0·08–0·11)	0		0·05 (0·04–0·06)	0·02 (0·01–0·02)	0	
Chicken	0·05 (0·04–0·06)	0·02 (0·02–0·03)	0·03 (0·02–0·03)	0	0·03 (0·02–0·03)	0·00*	0·00*	0
	F_{st} MLST				F_{st} *flaB*			
	Cattle	Human	Chicken	Pig	Cattle	Human	Chicken	Pig
C. coli								
Cattle	0				0			
Human	0·23 (0·11–0·31)	0			0·14 (0·12–0·16)	0		
Chicken	0·08 (0·04–0·11)	0·04 (0·00–0·08)	0		0·08 (0·07–0·10)	0·00#	0	
Pig	0·05 (0·01–0·07)	0·27 (0·14–0·39)	0·18 (0·10–0·25)	0	0·12 (0·11–0·13)	0·39 (0·36–0·41)	0·35 (0·32–0·37)	0

Values within parentheses indicate F_{st} bootstrap 2·5 and 97·5 percentile values (over 20 000 bootstraps).
0 = maximal similarity; 1 = maximal difference.
* Not significantly different from 0.

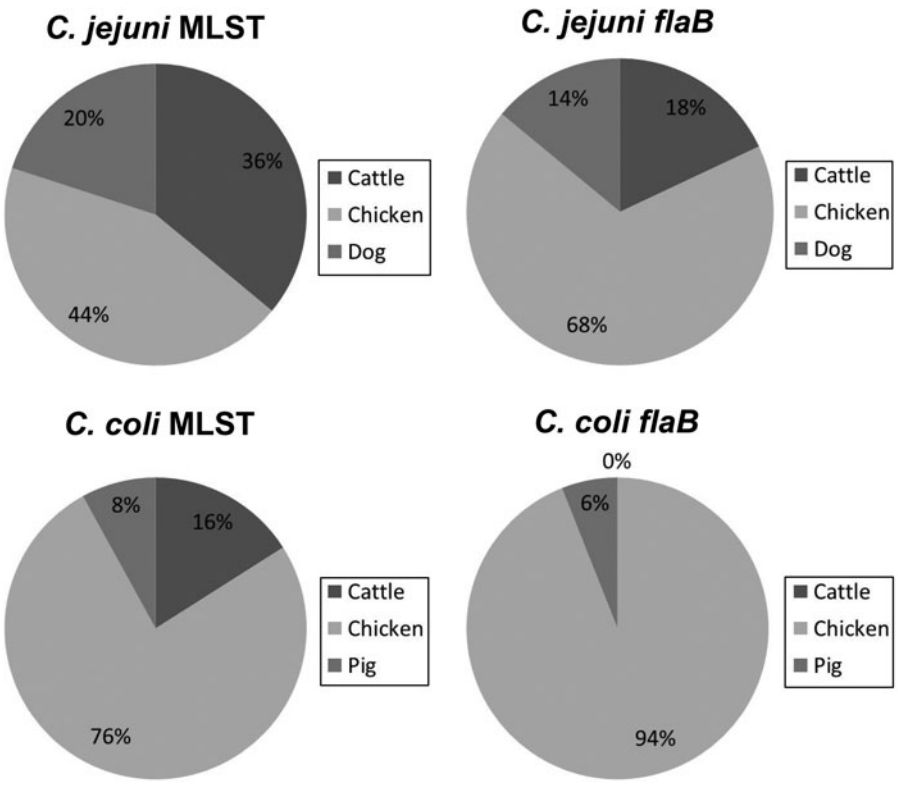

Fig. 1. Source assignment of human *Campylobacter* isolates to the cattle, chicken, dog and pig reservoir using STRUCTURE software.

while for *C. coli* highest similarity was observed with chicken isolates. Again human isolates were always closest to chicken isolates for both *Campylobacter* sp. independent of the typing data used. In the case of *flaB* sequences, the genetic distance between human and chicken isolates did not even differ significantly from zero.

Source attribution

The rank of source attribution based on STRUCTURE analysis for human *C. jejuni* isolates was the same with both MLST and *flaB*. More human isolates were attributed to chicken than to cattle and the least to dogs; they reached 44%, 36% and 20%, respectively, for MLST and 68%, 18% and 14% for *flaB* typing. Concerning human *C. coli*, the analysis using MLST data revealed a similar ranking with chicken (76%), followed by cattle (16%) and pigs (8%). With *flaB* typing in *C. coli*, the results differ from the others as no human isolates were assigned to cattle, but almost all isolates were assigned to chicken (94%) and a few to pigs (6%) (Fig. 1).

DISCUSSION

This is the first study to investigate the population structure of *C. jejuni* and *C. coli* in Swiss cattle. A multiplex approach including MLST, *flaB* typing and genetic determination of antibiotic resistance to quinolones and macrolides was applied. The proportion of *C. jejuni* was higher than *C. coli* with 75% and 25%, respectively. This corresponds to findings from other studies [34, 35]. However, *C. coli* prevalence in Switzerland was higher than that reported by Sproston *et al.* [9] at 2·3% in the UK. This variation in *C. coli* frequencies may be related to the specific farming structure in Switzerland, with farms having both cattle and pigs allowing contact between them. This hypothesis is supported by the comparatively high PSI results between cattle and pigs with MLST (0·36) as well as with *flaB* (0·47) data.

A great ST variety was observed in *Campylobacter* within the Swiss cattle population. The 97 investigated isolates contained 37 different STs of which two STs had not been previously described. The most common STs represented in our dataset (ST21, ST61, ST48, ST854) were also the most commonly reported STs in cattle in other countries [9, 34, 36].

As previously shown for other hosts, *flaB* typing demonstrated a higher discriminatory index than MLST for cattle isolates and if the two methods are combined, it increases the discriminatory power of each [25].

Further, with the inclusion of cattle isolates, population genetics analyses confirmed chicken as the major source for human campylobacteriosis in Switzerland as is the case for other countries [14–16, 19]. In fact, using *flaB* typing, the similarity between human and chicken isolates did not significantly differ from zero, indicating a high overlap of these two *Campylobacter* populations. Nevertheless, cattle seem to harbour *Campylobacter* populations similar to chicken and humans. Furthermore, ST61, which is typical for ruminants, was found for about 17% of cattle *C. jejuni* isolates in the UK [34], which is similar to the 16% determined in this study. ST61 is also found in about 1% of Swiss human *C. jejuni* isolates and cattle are a likely source for infection with this ST. A possible role of cattle as a source for human campylobacteriosis is further supported by the attribution of 36% of human *C. jejuni* to bovine *C. jejuni* based on MLST which is comparable to findings by other studies [14–16]. Interestingly, the source attribution of cattle *C. jejuni* as a source of infection for humans using *flaB* typing was less at only 18%. This difference could be due to higher mutation rates in the *fla* genes than in the housekeeping genes used for MLST which are under constantly high selection pressure.

Our analyses indicated 31% of cattle strains being resistant to quinolones, and only 1% resistant to macrolides (represented by only one *C. coli* strain). Similar rates of resistance were found in Switzerland for *Campylobacter* isolated from other animal species like chicken, pig and dog [29–31, 37]. Antibiotic resistance is more pronounced in human isolates whereas macrolide resistance is virtually absent [5]. In 2009 almost 40% of strains in patients without a history of foreign travel were quinolone-resistant and this figure rose to 56% for those with a history of recent foreign travel [5]. Wirz *et al.* [37] observed significant associations between specific genotypes and quinolones resistance/sensitivity in chicken isolates. Such an association was also found in cattle isolates with CC61 being significantly more often sensitive towards quinolones. This is a novel observation and it will be interesting to see if this is the case in other countries also.

In conclusion, *C. jejuni* and *C. coli* from Swiss cattle showed a high genetic diversity, with two new sequence types and five new *flaB* types discovered. Source attribution indicates that cattle should not be underestimated as a potential origin for human campylobacteriosis. Improvement regarding the high quinolone resistance status should be achieved to decrease its frequency.

ACKNOWLEDGEMENTS

This work was supported by the Federal Food Safety and Veterinary Office grant 1·10·08.

DECLARATION OF INTEREST

None.

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